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NITRATE REDUCTASE ACTIVITY AND NITROGENOUS GAS EVOLUTION FROM HETEROTROPHIC, PHOTOMIXOTROPHIC AND PHOTOAUTOTROPHIC SOYBEAN SUSPENSION CULTURES

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SUMMARY

Soybean (*Glycine max* L. Merr. cv. Corsoy) suspension cultures were grown hetertrophically (3% sucrose, dark), photomixotrophically (1% sucrose, light), and photoautotrophically (no sucrose, light) to determine if constitutive and inducible nitrate reductase (NR) activities were expressed (both activities are expressed in intact plants). Nitrogenous gas evolution, a marker for the constitutive activity in soybean leaves, was not detected in any of the cultures at any time during the 14-day subculture period. Moreover, photomixotrophic and heterotrophic cells grown on glutamine as a nitrogen source had no detectable NR. Cells grown on nitrate contained NR activity with an apparent K_m (NO_3^-) identical to that of the inducible activity from soybean leaves, and glutamine did not repress this activity. It was concluded that constitutive NR was not expressed in soybean suspension culture regardless of age, nitrogen source, or photosynthetic capability, while inducible NR activity was expressed with nitrate nutrition. The implications of these findings, in terms of using suspension cultures to select NR deficient soybean lines for possible regeneration, are discussed.

Key words: Glycine max — Soybean — Nitrate reductase — Nitrogen metabolism — Plant cell culture

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Abbreviation: NR, nitrate reductase.

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INTRODUCTION

Young leaves and cotyledons of soybean (*Glycine max* L. Merr. cv. Williams) plants contain both constitutive and inducible NR activities when grown on NO_3^--N (Ref. 1 and unpublished data). Nitrogenous gas evolution, noted during in vivo NR assays, is associated with constitutive NR activity and serves as a marker for this activity [1,2]. A mutant line (LNR-2) has been selected at the whole plant level, via ClO_3^- screen, which lacks the constitutive NR activity and the concomitant nitrogenous gas evolution [1]. Although efforts are continuing to select a totally NR deficient soybean at the whole plant level, screening at the cell level would be desirable since large numbers of cells can be quickly screened.

Soybean suspension culture cells grown on B5 medium (25 mM NO_3^- and 1 mM NH_4^+) exhibited maximum NR activity 6 days after subculture [3]. No differentiation between constitutive and inducible NR activities was made. Oaks [4] found that cells grown on 20 mM glutamine did not have measurable NR activity. Since subculture of these glutamine-grown cells into 25 mM KNO_3 plus 10 mM glutamine did not result in appearance of NR activity it was suggested that glutamine was actively repressing NR activity [4]. However, Bayley et al. [5] showed that addition of 5 mM glutamine to cells subcultured into B5 medium minus 1 mM NH_4^+ actually increased NR activity above the B5-grown controls.

Recently, Horn et al. [6,7] have described a system for growing soybean cells (cv. Corsoy) photoautotrophically, photomixotrophically and heterotrophically. In the study reported here all three types of growth conditions were utilized to determine (1) whether both constitutive and inducible NR activities are expressed in culture, (2) whether glutamine affects the appearance of constitutive and inducible NR activities in culture, and (3) whether photosynthetic activity in culture affects NR activity.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv. Corsoy) cell suspensions (derived from cotyledons) were subcultured every 14 days into their respective media. SB-P cells (photoautotrophic) were cultured as described by Horn et al. [6,7] in KT medium lacking sucrose under light (200—300 $\mu E\ m^{-2}\ s^{-1}$) and aerated aseptically with 5% (v/v) CO_2 in air. SB-M cells (photomixotrophic) were grown in KT medium containing 1% (w/v) sucrose under light. SB-H cells (heterotrophic) were grown on KT medium containing 3% (w/v) sucrose in complete darkness. When the medium contained filter sterilized glutamine (10 mM) as the sole nitrogen source, potassium was supplied as 35.8 mM KCl. In vivo NR activity and nitrogenous gas evolution were determined by the method described by Harper [8] with the following modifications. Assays were performed only in the presence of 50 mM KNO_3 using 0.5 g fresh wt. material. The fritted glass tubes

remained in the same trapping solution for 30 min. Nitrite present in the assay medium was determined after cell removal by filtration.

Kinetic analyses on SB-H and SB-P cells were conducted by the previously described method for in vitro NR analysis after liquid N_2 extraction [9], modified as follows. The extract mix did not contain KNO_3. Cell extracts (1.5 g fresh wt. per 10 ml extraction mix) were filtered through miracloth before centrifugation. Supernatant (3 ml) was passed through a Sephadex G-25 column (40 ml vol., 1.3 cm diameter, pre-equilibrated with extraction mix) to remove endogenous tissue NO_3^-. The Michaelis constant (K_m) for NO_3^- was obtained using the G-25 effluent and varying the NO_3^- concentration in the reaction mix from 0.075 mM to 5 mM with a minimum of seven intermediate NO_3^- concentrations. The reaction mix contained 16 mM potassium phosphate, 0.63 μM FAD, 13.2 mM K_2CO_3, 0.37 mM NADH (saturating), and 0.5 ml enzyme in a final volume of 4.75 ml (final pH 7.0). The reaction was started by addition of pyridine nucleotide. Reactions (30°C) were terminated by mixing of 1 ml subaliquots with 212 μl of 0.25 M Zn-acetate. Reaction velocities were calculated from the 8—16 min time interval when linearity had been established. Apparent K_m (NO_3^-) values were determined via Eadie-Hofstee plots. Each value was obtained from a minimum of duplicate analyses.

RESULTS

Heterotrophic cells

SB-H cells grown in the dark on medium containing 3% (w/v) sucrose lack chlorophyll and are thus incapable of light-dependent CO_2 fixation. These cells, grown on Murashige-Skoog [10] medium (NO_3^- + NH_4^+), had in vivo NR activity which peaked at 3—6 days after subculture (12 μmol NO_2^- g fresh wt.$^{-1}$ h^{-1}, Fig. 1). The apparent K_m for NO_3^- was approx. 0.16 mM for NR in crude extracts from cells 7 days after subculture. Glutamine-grown cells contained less than 1% of the (NO_3^- + NH_4^+)-grown cell in vivo NR activity and were frequently zero. Neither (NO_3^- + NH_4^+)- nor glutamine-grown cells exhibited substantial nitrogenous gas evolution (Fig. 1).

To determine whether glutamine was repressing inducible NR activity, SB-H cells, previously grown on a glutamine nitrogen source for 1 month, were inoculated into media containing (NO_3^- + NH_4^+) in addition to 10 mM glutamine (Fig. 2). NR activity was induced within 1 day to a higher level than cells previously grown on (NO_3^- + NH_4^+) medium and subcultured into the same medium (5.80 vs. 4.38 μmol NO_2^- g fresh wt.$^{-1}$ h^{-1}). In addition, when 10 mM glutamine was added to 5-day-old cells growing on (NO_3^- + NH_4^+) medium there was no effect on the in vivo NR activity of these cells compared with the (NO_3^- + NH_4^+) control cells. Cells transferred from glutamine medium to a medium containing no nitrogen supply showed no appreciable increase in NR activity before exhibiting nitrogen starvation symptoms and eventual death (data not shown).

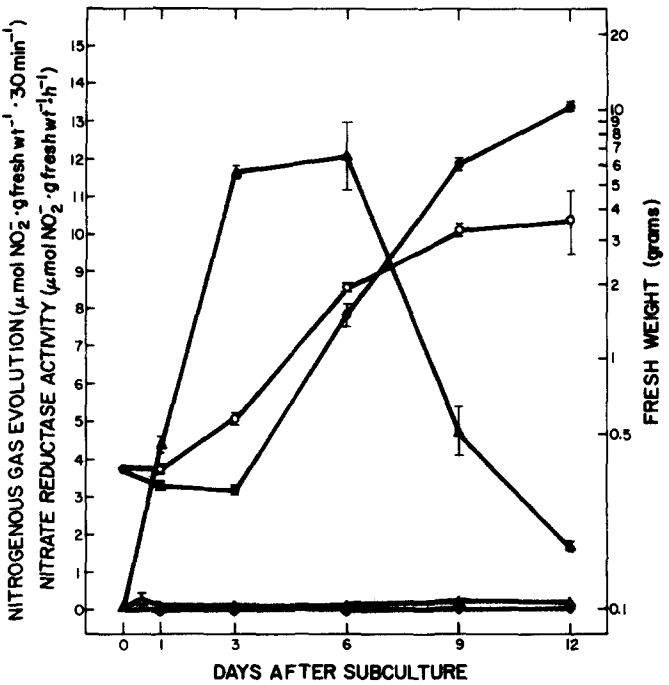

Fig. 1. Time course of fresh weight, nitrate reductase and nitrogenous gas evolution of heterotrophic soybean cells growing on KT-3% (3% sucrose) medium containing (NO_3^- + NH_4^+) as nitrogen source (filled symbols) and containing glutamine as the sole nitrogen source (open symbols). Fresh weight (●——●, ○——○); in vivo NR activity (▲——▲, △——△); nitrogenous gas evolution (both nitrogen treatments) (◆——◆). Data are means of two replicates ± S.D.

Photomixotrophic cells

SB-M cells grown on (NO_3^- + NH_4^+) medium with 1% (w/v) sucrose obtain approx. one-third of their carbon from CO_2 and the other two-thirds from sucrose [7]. As shown in Fig. 3 they evolved only trace levels of nitrogenous gas when grown on (NO_3^- + NH_4^+) medium or on medium containing glutamine as the sole nitrogen source. As in the case of SB-H cells, SB-M cells grown on (NO_3^- + NH_4^+) medium exhibited a peak of NR activity at 6 days after subculture (12.2 μmol NO_2^- g fresh wt.$^{-1}$ h^{-1}) while cells grown on glutamine exhibited almost no NR activity at any stage of the growth cycle (lag, logarithmic, and stationary phases, Fig. 3). Glutamine-grown cells exhibited light-stimulated $^{14}CO_2$ fixation, when determined by the method described by Horn et al. [7], although chlorophyll levels were decreased by approx. 50% when compared to the (NO_3^- + NH_4^+)-grown controls (data not shown).

Photoautotrophic cells

SB-P cells depend entirely on CO_2 for their carbon supply and as such are more similar to isolated leaf cells than are standard heterotrophic cell

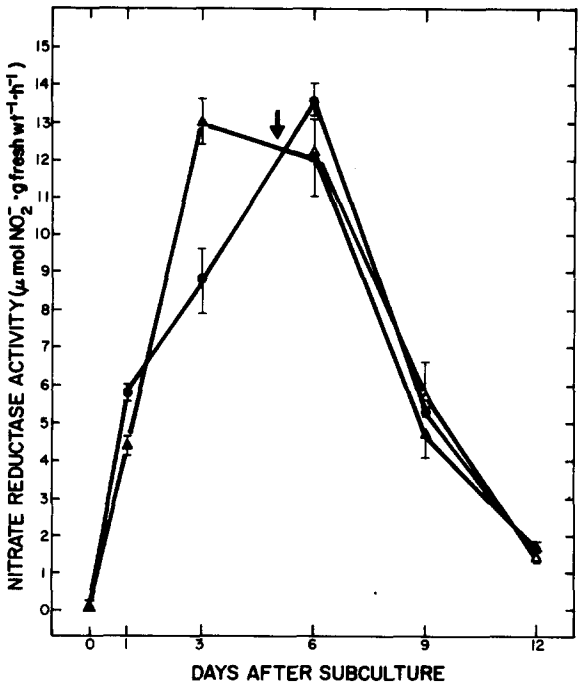

Fig. 2. Effect of glutamine on in vivo nitrate reductase activity in heterotrophic soybean cells. The cells were (1) grown on KT-3% medium with glutamine as the sole nitrogen source for 1 month (two subcultures) and then transferred to KT-3% medium containing 40 mM NO_3^-, 20 mM NH_4^+ and 10 mM glutamine (●——●); or (2) grown on KT-3% with (NO_3^- + NH_4^+) as nitrogen source and then 10 mM glutamine was added on day 5 (arrow). No glutamine (▲——▲); added glutamine (△——△). Data are means of two replicates ± S.D.

cultures. When examined for NR activity over time (Fig. 4), a distinct peak at 6 days after subculture was evident (13.6 μmol NO_2^- g fresh $wt.^{-1}$ h^{-1}). The apparent K_m for NO_3^- was approx. 0.19 mM for NR in crude extracts from cells 8 days after subculture. At no time did the nitrogenous gas evolution exceed trace amounts (Fig. 4). These cells were not grown with glutamine as the sole nitrogen source since it could also serve as a carbon source, thus changing the SB-P cells into SB-M cells. Urea, another alternative reduced nitrogen source, caused rapid bleaching in both SB-P and SB-M cells (data not shown).

DISCUSSION

Initial experiments, conducted on whole plants, indicated that constitutive NR activity and the associated nitrogenous gas evolution from young leaves of Corsoy were comparable to that previously reported for Williams [1]. Although Corsoy plants expressed constitutive NR activity

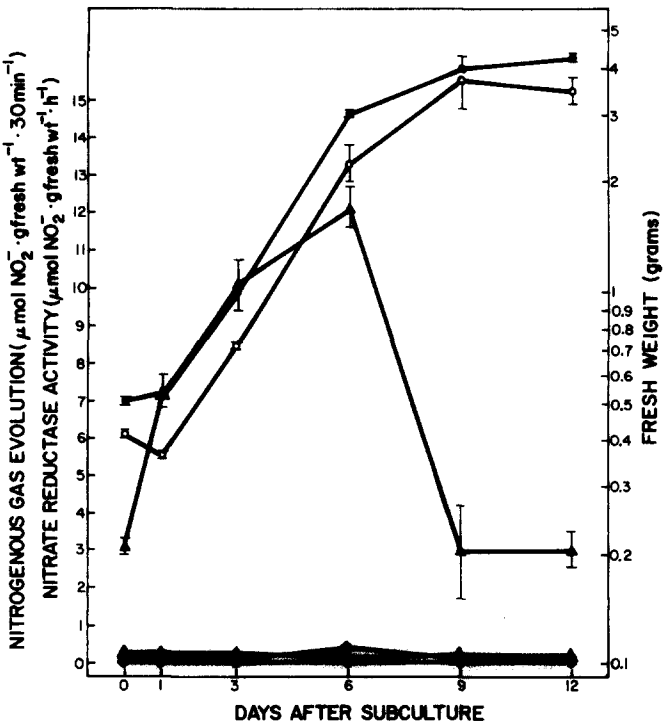

Fig. 3. Time course of fresh weight, nitrate reductase activity and nitrogenous gas evolution of photomixotrophic soybean cells growing on KT-1% (1% sucrose) medium containing NO_3^- + NH_4^+ as nitrogen source (filled symbols) and containing glutamine as the nitrogen source (open symbols). Fresh weight (●——●, ○——○); in vivo NR activity (▲——▲, △——△); nitrogenous gas evolution (◆——◆, ◇——◇). Data are means of two replicates ± S.D.

and nitrogenous gas evolution, none of the cell types (heterotrophic, photomixotrophic, or photoautotrophic) evolved more than trace amounts of nitrogenous gas (less than 0.3% that evolved by young soybean leaves), regardless of nitrogen source ((NO_3^- + NH_4^+) or glutamine) (Figs. 1, 3 and 4). The (NO_3^- + NH_4^+)-grown cells exhibited in vivo NR activity but this appeared to be strictly inducible NR activity since only trace levels of nitrogenous gas were evolved; nitrogenous gas being a marker of constitutive NR activity [1,2]. In addition, the Michaelis constant for NO_3^- displayed by NR from SB-H (0.16 mM) and SB-P (0.19 mM) cell extracts, were similar to that for NR from crude extracts of the soybean mutant, LNR-2, lacking constitutive NR activity and different from that for NR from crude extracts of wild-type (cv. Williams) plants containing only constitutive NR activity and assayed under identical conditions (data not shown). The glutamine-grown cells had no in vivo NR activity (Fig. 1 and 3) which as Oaks [3] suggested may have been due to active repression of NR activity by this nitrogen source. However, addition of (NO_3^- + NH_4^+) to glutamine-grown

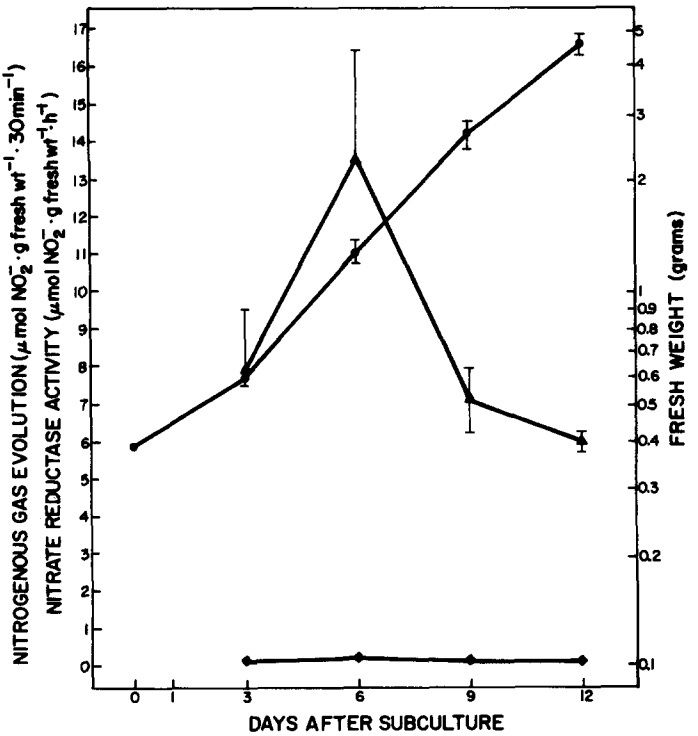

Fig. 4. Time course of fresh weight, nitrate reductase activity and nitrogenous gas evolution of photoautotrophic soybean cell cultures. Cells were grown on KT-0% (no sucrose) medium containing (NO_3^- + NH_4^+) as nitrogen source. Fresh weight (●——●); in vivo NR activity (▲——▲); nitrogenous gas evolution (◆——◆). Data are means of two replicates ± S.D.

cells resulted in NR activity equivalent to cells grown on (NO_3^- + NH_4^+) alone (Fig. 2). Also addition of glutamine to (NO_3^- + NH_4^+)-grown cells 5 days after subculture did not inhibit NR activity in these cells (Fig. 2). These results indicated that 10 mM glutamine, in the presence of 40 mM NO_3^--N and 20 mM NH_4^+-N, was not actively repressing inducible NR activity as also noted by Bayley et al. [5]. Fukunaga and King [11] analyzed suspensions of *Datura innoxia* (Mill.) cells and they also showed an increase in NR activity above controls, after addition of 1 mM glutamine to cells growing on 25 mM KNO_3. It thus seemed unlikely that constitutive NR activity was repressed by glutamine, especially when one considers that this activity appeared to be present in only trace amounts even in a non-repressive medium (NO_3^- + NH_4^+), as measured by nitrogenous gas evolution.

In whole plants constitutive NR activity was present only in young green tissue [1], indicating that photosynthesis may be important for

the initiation of this activity. However, neither photoautotrophic nor photomixotrophic cells, shown to have light-stimulated CO_2 uptake [6,7], exhibited constitutive NR activity (Fig. 3 and 4). Therefore, photosynthesis alone was not the key to expression of constitutive NR activity. A similar conclusion was drawn by Duke et al. [12] using cotyledons from whole plants.

It is concluded that constitutive NR activity is not expressed in these soybean suspension cultured cells regardless of nitrogen source or photosynthetic capability. These findings suggest that cultured cells of commercial cultivars selected for total NR deficiency, by ClO_3^- screen for example, may still express constitutive NR activity in the whole plant, assuming successful regeneration. Thus, cell lines derived from whole plants already lacking constitutive NR activity (e.g., LNR-2; Ref. 1) may be better candidates for the cell-level search for totally NR deficient plants.

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